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nt women [5], and when an excessive thrombin generation is present, trophoblast apoptosis may occur [6].

A normal pregnancy is dependent on adequate placental circulation and foetal vasculature. An adequate balance between coagulation and fibrinolysis is mandatory to avoid an excessive fibrin accumulation in placental vessel and intervillous space, as well as a greater propensity to undergo partial or total occlusion of placental vessels by thrombus formation [7]. Data from clinical studies provided evidence for an association between increased factor VIII (FVIII) concentrations and recurrent abortion [8], as well as hypofibrinolysis, mainly related to high PAI-1 levels, is involved in unexplained early recurrent miscarriage [9]. Therefore, these parameters might be used in exploring a potential prothrombotic status in women suffered from RPL.

A prothrombotic status may determine an exaggerated response during pregnancy, that in turn may affect both pregnancy outcome and future women health. To date, it

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has been demonstrated that negative pregnancy outcome, such as unexplained RPL, represent an independent risk factor for subsequent long-term cardiovascular and cerebrovascular morbidity, in particular, subsequent risks of myocardial infarction (MI), cerebral infarction and renovascular hypertension [10,11]. Interestingly, data from literature showed that, even when traditional cardiovascular risk factors, such as hypertension, dyslipidaemia, smoking habit, BMI, diabetes and cardiovascular family history, are accounted for, RPL was associated with an increased risk of arterial thrombosis (ischemic stroke and MI) [12]; all these findings represent the rationale for risk stratification and suggest preventive intervention through a later follow-up of healthy women suffering pregnancy losses.

Coagulation assays traditionally used, such as FVIII and PAI-1, may be inadequate to assess the overall thrombotic potential of subjects investigated; the evaluation of thrombin production and activity through global coagulation assay, such as calibrated automated thrombography (CAT), could better highlight the presence of a procoagulable status with respect to a traditional coagulation protein assay [13]. Moreover, a correlation between single coagulation factors, such as fibrinogen, and the global thrombin potential performed by CAT assay has also been reported [14].

At the best of our knowledge, only two studies investigated the coagulation pathway by CAT assay in women with history of unexplained RPL [13,15], reporting conflicting results. In this scenario we evaluated hemostatic profile by global and traditional coagulation assays in healthy women with history of RPL compared with uneventful pregnancy women, to search for a possible relationship between an underlying procoagulant status and RPL.

Methods

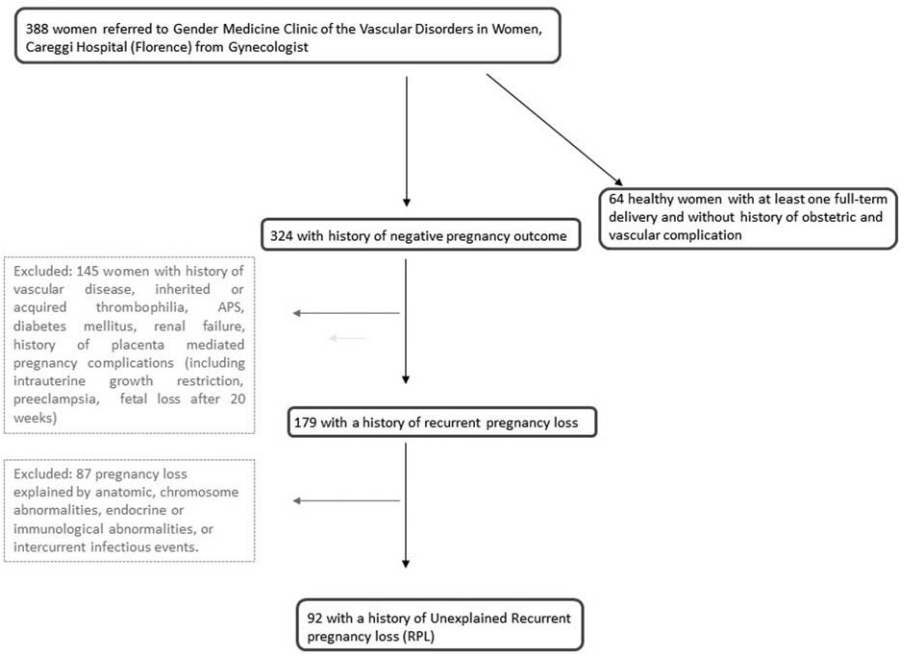
Study population

The entire study population comprised 388 consecutive white not pregnant women referred to Gender Medicine Clinic for the Vascular Disorders in Women, Careggi Hospital from 2014 to 2016, to be framed for vascular risk. In Fig. 1, the flow chart of the study population was reported.

Ninety-two out of 388 (23.7%) women experienced unexplained RPLs defined as three or more consecutive pregnancy losses with the same partner before 20 weeks of gestation; information concerning the adverse obstetrical outcome derived from written gynaecologists' clinical report.

Sixty-four women (uneventful pregnancy women) who had never had unexplained RPL and/or other pregnancy complications, who delivered after uneventful pregnancy (at least one living child), referred to Gender Medicine Clinic for evaluating thrombotic risk before taking estrogen-progesterone therapy or for family history of vascular disorders, were considered as controls.

Fig. 1



Flow-chart of study population.

To identify disease free controls, and to exclude women who were thought to have any form of vascular disease (venous thromboembolism or arterial disease) and familial history of cardiovascular disorders, a detailed interview was performed.

All women were investigated for hemostasis and thrombin generation parameters at the follicular phase, after a minimum of 6 months after events. None was pregnant or using oral contraceptive from at least 8 weeks before testing. Exclusion criteria were: history of vascular disease (venous thromboembolism or arterial disease), the presence of inherited and acquired thrombophilia, antiphospholipid syndrome, diabetes mellitus, renal failure, history of placenta mediated pregnancy complications (including intrauterine growth restriction, preeclampsia, foetal losses after 20 weeks). Pregnancy loss explained by anatomical abnormalities possibly contributing to pregnancy failure, chromosome abnormalities (maternal or paternal carrier of a structural chromosomal rearrangement) and endocrine or immunological abnormalities, or intercurrent infectious events, were also excluded. Informed written consent for anonymous data analysis was obtained from each woman. The investigation conformed with the principles outlined in the Declaration of Helsinki.

Coagulation parameters

Blood samples were collected from the antecubital vein into 0.109 mol/l trisodium citrate tubes (Vacutainer, Becton Dickinson, New Jersey, USA) in the morning, after an overnight fasting. Plasma samples were obtained by centrifuging blood at $2000 \times g$ for 15 min at room temperature for von Willebrand factor (vWF)-ag, fibrinogen, FVIII and at 4 °C only for PAI-1 evaluation. Complete blood cell count was performed by using the Sysmex XE-2100 hematology analyzer (Sysmex, Kobe, Japan). Fibrinogen was assessed by Clauss clotting method (Siemens, Marburg, Germany). Plasma PAI-1 antigen levels were measured with commercially available ELISA kits (PAI-1 Actibind ELISA, Technoclone, Wien, Austria). FVIII activity was determined by a coagulation-based assay, with deficient plasma in the presence of Pathromtin (Coagulation Factor VIII, Siemens); vWF antigen levels were detected by a turbidimetric assay (vWFAg, Siemens).

Calibrated automated thrombography

Blood samples for CAT analysis were centrifuged at $2000 \times g$ for 15 min at room temperature. After removal of the cells, plasma was centrifuged for a second time at the same setting; platelet poor plasma (PPP) was stored at −80 °C until use.

We used the method described by Hemker *et al.* [16] and commercialized by Thrombinoscope BV (Maastricht, the Netherlands). According to the manufacturer's instructions, measurements were conducted in 80 μl of PPP triggered by 20-μl PPP-reagent (tissue factor 5 pmol/l) in 96-well microtitre plates. Measurements were calibrated against the fluorescence curve obtained in the same plasma mixed with 20-μl thrombin calibrator (Thrombinoscope BV). Fluorogenic substrate (20 μl) were added to sample and calibrator wells, and the fluorescence intensity was detected in a Fluoroskan Ascent reader (Thermo Labsystems OY, Helsinki, Finland) with a 390/460 filter set for 60 min, and the thrombin generation curves were calculated with Thrombinoscope software (Thrombinoscope BV).

Thrombin generation was expressed as endogenous thrombin potential (ETP: the area under the curve that represents the total amount of thrombin generated), peak height (the maximum concentration of thrombin produced), velocity index (the slope between the start of thrombin formation and the peak), lag time (the time in minutes from initiation of the test to the start of thrombin formation) and time to peak (the time in minutes to generate the highest point on the curve).

Statistical analysis

Statistical analysis was performed by using the SPSS software (Chicago, Illinois, USA) for Windows (Version 11.5, Microsoft, Washington USA).

Age and continuous variables were expressed as median (range), and the categorical variables were expressed as frequencies and percentages. Continuous variables were analysed using independent samples *t* test or Mann–Whitney as appropriate, and the categorical variables with chi-square test.

To identify a possible cut-off value of ETP concentration, we divided our study population into quartiles of controls ETP distribution (first quartile: <1102.4; second quartile: 1102.5–1222.0; third quartile: 1222.1–1465.1; fourth quartile: >1465.2 nmol/l). Mann–Whitney test was performed to compare quartile distribution of ETP between patients and controls. To analyse the ETP cut-off accuracy, the ROC curve analysis was performed.

A logistic regression analysis was used to evaluate the role of ETP cut-off in modulating RPL risk. Odds ratios and 95% confidence intervals (CIs) were presented. A *P* value less than 0.05 was considered to indicate statistical significance.

Correlation analysis was measured by using the Spearman's correlation test, and post-hoc multiple comparison analysis was performed by using Tukey test.

Sample size calculation was based on data derived from Bennett *et al.* [13]; in this study, by assuming a difference of 10% in peak height between patients and controls, no significant difference was found. Therefore, we hypothesized that difference in peak height would be lower than 10%; by using their controls peak height values (284.8 ± 48.7), a sample size of at least 58 women for each group was deemed sufficient to detect a difference

Table 1 Demographical and Clinical characteristics of the study population

	Women with RPL, $n=92$		Control women, $n=64$		P
Demographic and clinical parameters					
Age[a]	38	(26–42)	37	(25–40)	0.3
	n	%	*n*	%	
Smoking habits	16	17.4	11	17.1	0.5
BMI > 25 kg/m^2	26	28.3	11	17.2	0.07
Familial history of CV disease	25	27.2	6	9.3	**0.005**
Women with history of pregnancy loss	92	100	0	0	
Three previous pregnancy loss	67	72.8		–	
Four previous pregnancy loss	18	19.6		–	
Five previous pregnancy loss	7	7.6		–	
Gravidity	386		102		
Number of pregnancy loss	308	79.8	0	0	
Live birth	78	20.2	102	100	**0.0001**

CV, cardiovascular; RPL, recurrent pregnancy loss. Bold values indicate statistical significance. [a]Values are given as median (range).

of 9%, with a statistical power of 80% (β) and a significance value of 5% (α).

Results

In Table 1, demographic and clinical characteristics of the study population were shown. No significant difference in age and smoking habit between women with RPL and controls was observed; a higher prevalence of both BMI more than 25 kg/m^2, and familial history of cardiovascular disease was observed in women with history of RPL in comparison with that found in controls.

Among 92 women with history of pregnancy loss, 67 (72.8%) experienced three events, 18 (19.6%) four events and 7 (7.6%) have had five consecutive losses (Table 1).

As concerns, haemostatic parameters investigated, higher fibrinogen, FVIII and PAI-1 levels in women with RPL compared with controls were observed; no difference in vWF concentration between patients and controls was found (Table 2).

By evaluating thrombin generation through the parameters deriving from the CAT assay, we observed that ETP and peak height were significantly higher in RPL as compared with controls; moreover, a significant lower lag time in RPL with respect to controls was found (Table 2).

By exploiting ETP median values, we observed a significant different distribution between RPL patients and controls ($P=0.001$).

To identify a cut-off of ETP concentration, which could be associated with a high RPL risk, we divided our study population into quartiles of controls ETP distribution. In Fig. 2, the percentage of both RPL women and controls, according to ETP quartiles, is reported; in particular, in the first ETP quartile, we found the same percentage of patients and controls, and in the second quartile a lower percentage of RPL with respect to control women was observed (second quartile: 33.3 vs. 66.7%, respectively); a higher percentage of RPL in comparison with control women in third and fourth quartile was found (third quartile: 59.0 vs. 41.0%, respectively; fourth quartile: 70.6 vs. 29.4%, respectively) ($P=0.009$) (Fig. 2). Based on these results and according to controls ETP distribution, ETP concentration of 1222.1 nmol/l was considered as cut-off value.

At the ROC curve analysis, a high specificity and sensibility of the cut-off value was evidenced (Area Under Curve = 0.63, $P=0.009$).

Accordingly, the potential role of ETP cut-off value in predicting RPL risk was investigated; our results showed that ETP concentration above cut-off value was associated with more than two-fold increased risk of RPL [Odds Ratio (OR) = 2.56, CI; 1.28–5.13, $P=0.008$], also after adjustment confounding variables and traditional cardiovascular risk factors (smoking habit, BMI > 25, familial history of cardiovascular disorders) (OR = 2.63, CI; 1.28–5.41, $P=0.009$).

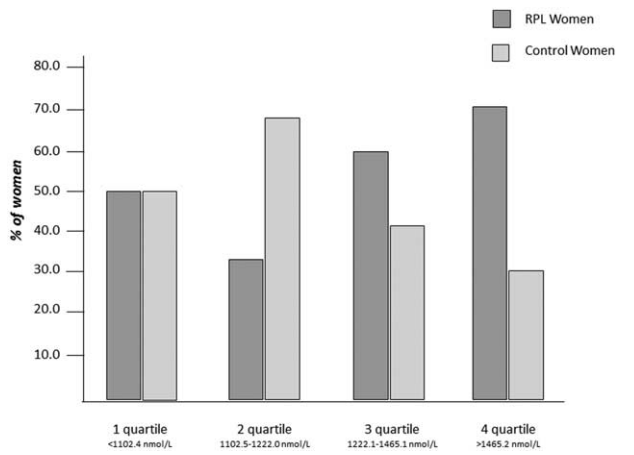
We also investigated the relationship between thrombin generation and hemostatic parameters known to affect vascular environment, such as FVIII, PAI-1 and Fibrinogen (Table 3); our findings showed a positive relationship between FVIII and PAI-1 and thrombin generation; in particular, FVIII significantly correlated with ETP, peak and velocity index in RPL women, but not in controls,

Table 2 Hemostasis and thrombin generation parameters among recurrent pregnancy loss and controls women

	Women with RPL, $n=92$	Control women, $n=64$	P
Hemostasis parameters			
vW factor[a]	106.0 (40.2–253.0)	104.2 (46.5–188.0)	0.9
Fibrinogen (mg/dl)[a]	405 (224–549)	320.5 (236–415)	**<0.0001**
FVIII[a]	105.8 (46.5–283.0)	90.5 (47.0–140.0)	**0.04**
PAI-1 (ng/ml)[a]	21.6 (12.8–68.0)	7.0 (2.0–48.0)	**<0.0001**
Thrombin generation parameters			
Lag time (min)[a]	2.67 (1.67–4.33)	2.61 (1.64–4.32)	**0.05**
ETP (nmol/l min)[a]	1412 (551–2853)	1222 (840–1966)	**0.009**
Time to peak (min)[a]	5.83 (4.00–11.67)	6.00 (3.98–8.50)	0.8
Peak (nmol/l)[a]	244 (46–507)	209 (78–413)	**0.04**
Vel index (nM/min)[a]	76.1 (8.0–236.0)	63.5 (17.7–1777.0)	0.1

RPL, recurrent pregnancy loss. Bold values indicate statistical significance. [a]Values are given as median (range).

Fig. 2



Percentage of recurrent pregnancy loss women and control women among quartiles of endogenous thrombin potential distribution.

whereas PAI-1 significantly correlated with ETP in both RPL women and control group. A significant correlation between fibrinogen and lag time in both groups was found. After multiple comparison analysis, FVIII, but not PAI-1, still remained significantly correlated with both peak and velocity index in RPL women ($P = 0.001$ and <0.0001, respectively). As concerns control women, PAI-1 still remained significantly correlated with ETP, peak and velocity index ($P < 0.0001$).

Discussion

Our results evidenced that women with history of three or more consecutive unexplained pregnancy losses exhibited higher thrombin generation than women with uneventful pregnancy. This datum allow us to hypothesize a relationship between an underlying procoagulant status, related to both thrombin generation and altered hemostatic parameters, and negative obstetric events, such as unexplained RPL. Moreover, in this study, we define an ETP cut-off value, a measure of potential hypercoagulability, which might be used to frame RPL women risk profile, also in the presence of traditional risk factors.

Really, pregnancy loss is associated with altered hemostasis balance traditionally attributed to an excessive fibrin accumulation in placental vessel and intervillous space, as well as a greater propensity to undergo partial or total occlusion of placental vessels by thrombus formation [7]. Moreover, data from an experimental study suggest a further mechanism related to thrombin, which, by acting as cell mediator, increases trophoblast apoptosis and impairs trophoblast invasion [6]. Accordingly, the onset of a prothrombotic status could determine an altered trophoblast development, and at later gestational ages utero-placental vascular insufficiency [15]. Therefore, based on these mechanisms, we could hypothesize that high thrombin concentration, by acting both as signalling molecule and hemostasis component, may increase the risk of early or late pregnancy loss.

Thrombin levels are not only related to the amount of prothrombin, but also to the kinetic of prothrombin conversion reaction and thrombin inactivation; moreover, thrombin generation is modulated by several inhibitors and cofactors [17].

CAT assay, which measures the individual overall capacity to produce thrombin, is potentially more useful to evaluate hemostasis potential compared with conventional tests, such as prothrombin activation products (prothrombin fragment F1 + 2) and Thrombin antithrombin complex levels, which can identify only a hypercoaulable status already existing at full rate [18]. Our results showed increased ETP and peak parameters in RPL women, but also a longer lag time. This datum should be related to higher fibrinogen levels, as previously described [19]. Really, Dielis *et al.* have shown, in normal population, that fibrinogen may produce not only a procoagulant effect but also an anticoagulant effect by increasing lag time. This dual pro-coagulant/anticoagulant effect can be explained by the ability of fibrinogen to

Table 3 Correlation between thrombin generation and hemostasis parameters among recurrent pregnancy loss and controls women

	Lag time	ETP	Time to peak	Peak	Vel index
RPL women					
PAI-1	0.494	0.778	0.234	0.07	0.017
	$P=0.2$	$\mathbf{P=0.01}$	$P=0.5$	$P=0.8$	$P=0.9$
FVIII	−0.122	0.508	−0.365	0.561	0.557
	$P=0.4$	$\mathbf{P<0.0001}$	$\mathbf{P=0.005}$	$\mathbf{P<0.0001}$	$\mathbf{P<0.0001}$
Fibrinogen	0.336	−0.096	0.226	−0.129	−0.136
	$\mathbf{P=0.008}$	$P=0.4$	$P=0.08$	$P=0.3$	$P=0.2$
Control Women					
PAI-1	0.080	0.852	−0.176	0.693	0.534
	$P=0.5$	$\mathbf{P<0.0001}$	$P=0.2$	$\mathbf{P<0.0001}$	$\mathbf{P<0.0001}$
FVIII	−0.214	0.141	−0.203	0.099	0.120
	$P=0.3$	$P=0.5$	$P=0.4$	$P=0.6$	$P=0.6$
Fibrinogen	0.334	0.403	0.286	0.131	−0.065
	$\mathbf{P=0.08}$	$\mathbf{P=0.03}$	$P=0.1$	$P=0.5$	$P=0.7$

ETP, endogenous thrombin potential; RPL, recurrent pregnancy loss. Bold values indicate statistical significance.

bind to thrombin through binding sites, which are needed for the thrombin-mediated FVIII activation. This fibrinogen binding leads to an anticoagulant effect in the initiation phase, as also reported by Hemker *et al.* [14] who compared thrombin generation in full and defibrinated plasma.

At the best of our knowledge, only two studies were performed by using CAT assay in women with history of RPL [13,20], reporting conflicting results. Data from Bennett *et al.* [13] did not provide evidence of a procoagulant status in RPL women, even if an increase in ETP and peak height was found. Although several likenesses in both patients enrolment and exclusion criteria were present, some differences existed between our and data from Bennett, in particular ethnicity and timing of pregnancy losses; moreover, our study was conducted in a larger cohort of patients.

Another study, by investigating in RPL women the relationship between thrombin generation and pregnancy losses, evidenced enhanced thrombin generation, in particular when the assay was performed in the presence of thrombomodulin [20]. This datum is in keeping with our results in highlighting a procoagulant status in RPL women; however, our findings, obtained in the absence of thrombomodulin, and referred to a study population free from vascular events and thrombophilia, seem to be more notable. The addition of thrombomodulin could unmask alterations in protein C pathway, related to thrombotic events [21]; therefore, thrombin generation assay in presence of thrombomodulin is fitting in study population with history of vascular events, as is reported in the study from de Saint Martin.

To date, the evidence of hypercoagulability in RPL women, apart from clinical events, is supported by data from Rai *et al.* [15] who used another global coagulation assay, the thromboelastography. We did not perform thromboelastography assay, which is sensitive to cellular and plasmatic components and explores both clot formation and its breakdown, and this represent a limitation of our study. On the other hand, we evaluated both coagulation and fibrinolysis markers, such as fibrinogen, FVIII and PAI-1, demonstrating higher concentrations of these parameters in women with history of RPL, in keeping with data from global assays. Of interest, our results provided evidence for a significant correlation between FVIII levels and CAT parameters in patients, but not in controls, thus underling the role of this factor in maintaining a thrombophilic condition in RPL women outside of pregnancy, as previously reported [8,22].

Another limitation of our study is the lack of information concerning follow-up of women with history of RPL, as RPL represents an independent risk factor for subsequent long-term cardiovascular and cerebrovascular events [12]. Therefore, the identification of women with underlying hypercoagulable status, apart from the clinical event, might pave the way to a possible therapeutic approach in controlling their future risk.

Conclusion

In conclusion, we demonstrated, by using the global CAT assay, an underlying alteration of vascular network related to increased coagulation components, and fibrinolysis inhibitor levels in women with history of unexplained RPL and free from vascular events and thromophilic defects. Therefore, we could suggest that CAT global assay and routine testing for FVIII and PAI-1 should be used in clinical practice to evaluate the hypercoagulable status in healthy RPL women planning future pregnancy. If these data are confirmed in larger studies, it will be intriguing to speculate whether the hypercoagulable profile will be of value in the setting of antithrombotic strategies in unexplained RPL women.

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Conflicts of interest

There are no conflicts of interest.

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